SUPPLEMENTAL INFOMATION

Supplemental Materials and Methods

Neuronal and HEK cell culture and transfection

Primary cultured cortical neurons were prepared from embryonic day 18 rat embryos as previously described (Man et al. 2007; Hou et al. 2008a). Briefly, brain regions were dissected and digested with papain at 37℃. Dissociated neurons were seeded onto poly-L-lysine-coated coverslips at approximately $0.3x10^6$ cells per 60mm dish, each containing five coverslips. Neurons were maintained in Neurobasal medium (Gibco) supplemented with 2% B27, 1% horse serum, 1% penicillin/streptomycin and 0.4% L-glutamine for 2-3 weeks until use. 1 wk after plating, FDU (5 μM) was added to the media to inhibit glial. Transfections were performed at about 10-11 DIV and incubated for 4 h prior to media change. All cells were maintained in a humidified incubator at 37℃ in an atmosphere containing 5% CO_2. All transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Human embryonic kidney (HEK) 293A cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin and passaged at 100% confluency twice a week. Transfections were performed at approximately 50-70% confluency.

Lipofectamine 2000 was used for the transfection of both cultured neurons and HEK cells. Two days following transfection, cells were immunostained or lysed for biochemical analysis. For Nedd4 siRNA and viral ubiquitin double transfection experiments, cortical neurons were first transfected with siRNA, then infected 12 hrs later with ubiquitin virus for 48 hrs until experiments.

Plasmids and mutagenesis

On a previously described GFP-tagged GluA1 pRK5 construct (Man et al. 2007), the lysine (K) residues residing at amino acids 813, 819, 822 and 868 in the C-terminus were replaced with arginine (R) residues using Stratagene's QuikChange Site-Directed Mutagenesis kit. GFP-tagged GluA1 is approximately 125 kD. Mutations were verified by DNA sequencing. HA-tagged ubiquitin and Nedd4 cDNA were generously provided by Dr. Peter Snyder (University of Iowa).

Immunoprecipitation, western blotting and analysis

Cells were rinsed with cold PBS and resuspended in 100-200 μl modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1% SDOC and 0.1% SDS) containing mini cOmplete protease inhibitor (Roche) and 5 μM ubiquitin aldehyde (Sigma) to inhibit deubiquitination. Lysates were further solubilized by sonication and 10 minutes incubation on ice followed by centrifugation for 10 minutes at 13,000 x *g* to remove insolubilities. Supernatant volumes were adjusted to 500 μl with NP40 buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% NP40 plus mini cOmplete and 5 μM ubiquitin aldehyde) and incubated overnight for 8-12 hours on rotation at 4℃ with protein A-Sepharose beads (Santa Cruz Biotechnology) and antibodies against either GFP or GluA1. Immunocomplexes were washed 3 times with ice-cold NP40 buffer, resuspended in 2X Laemmli buffer and denatured on a 95℃ heat block for 10 min. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes and

probed with appropriate antibodies. Antibodies used for immunoblots were 1:500 HA α-Ms (ABM), 1:1000 Ub α-Ms (Sigma), 1:4000 β-tubulin α-Ms (Sigma), 1:500 GluA2 α-Rb (Millipore), 1:1000 GFP α-Ms (ABM), 1:1000 GluA1Ct α-Rb (Millipore) and 1:1000 Nedd4 (Abcam). For ubiquitination assays, all lysis buffers contained 1% SDS to avoid conventional protein-protein binding. Immunointensity of western blots were measured using Image J. For ubiquitination blots, smear signals above 100 kD were measured and quantified. GluA1 protein or GluA1 ubiquitination values were normalized to corresponding tubulin inputs where appropriate, then normalized to controls, prior to statistical analysis.

Immunoprecipitation of surface AMPARs

HEK 293A cells were transfected with GFP or GFP-GluA1 alone, or GFP-GluA1 together with HA-ubiquitin. 2 days after transfection cells were incubated with anti-GFP antibodies (1:100) for 10 min in culture medium at room temperature. After incubation, cells were washed three times with cold PBS on ice to remove free antibodies. Cell lysates were then incubated with protein A beads for 2 hours on rotation at 4℃. Following centrifugation, the pellets were reserved while the supernatants were then incubated with anti-GluA1Ct antibodies and protein A beads to isolate intracellular GFP-GluRs. The pellet eluates from both rounds of immunoprecipitation were subjected to SDS-PAGE gels and probed with anti-ubiquitin and anti-GluA1Ct antibody (Zhang et al. 2009).

Immunocytochemistry on AMPAR surface expression and ubiquitin

Transfected neurons were fixed for 10 minutes in an ice-cold solution of 4% paraformaldehyde, washed twice briefly with 1X ACSF (150 mM NaCl, 10 mM HEPES pH 7.4, 3 mM KCl, 2 mM $CaCl_2$, 10 mM glucose) and blocked for 1 hour in 10% NGS/ACSF. Coverslips were then incubated for 2 hours with primary antibodies (1:500) anti-GluA1Nt (Millipore) for endogenous AMPARs, or anti-GFP for transfected GFP-GluA1 in 10% NGS/ACSF, washed 3 times for 5 minutes with 1X ACSF and then incubated in the dark for 1 hour with Alexa Fluor-conjugated secondary antibodies (1:700). Coverslips were washed an additional 3 times for 5 minutes in the dark to remove unbound secondary antibodies before being mounted with ProlongGold Antifade (Invitrogen). For double staining, cells were fixed and permeabilized with 0.3% Triton X-100/ACSF for 10 minutes. Cells were then incubated with two sets of primary and fluorescence-conjugated secondary antibodies sequentially with three 5-min washes following antibody incubation.

Synaptosome preparation

Cortical tissue dissected from rat brains was minced and homogenized in either ice cold RIPA lysis buffer (for control lysate) or in ice cold synaptosome solution (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$) for synaptosome purification. Samples were then transferred to fresh 15 ml conical tubes and further solubilized by a half hour extraction at 4℃. The sample was then centrifuged at 1,400 x *g* for 10 mins. The supernatant (S1) was transferred to a new tube, and centrifuged at 13,800 x *g* for 10 mins. The remaining pellet (P2) containing the synaptosomes was then resuspended in RIPA lysis buffer. Protein amounts for both control and synaptosomal samples were obtained using the BCA protein determination kit (Thermo Scientific) and samples were diluted to the same protein concentration with RIPA lysis buffer. 2X Laemmli buffer was then added and samples were denatured on a 95℃ heat block for 10 min. Purity was further confirmed by western analysis.

Virus preparation

Full length human Nedd4.1 was PCR amplified to include the restriction sites NotI and BamHI using the following oligonucleotides:

5' GCGCGGCCGCATGGCAACTTGCGCGGTGGAG 3'

5' GCGGATCCCTAATCAACTCCATCAAAGCCCTGGG 3'

The PCR product was then gel-purified (QIAGEN QIAquick Gel Extraction Kit) and subcloned into the NotI and BamHI sites in the MCS region of the pHAGE-CMV-MCS-1ZsGreenW vector to create pHAGE-Nedd4.1. NotI and BamHI sites were regenerated.

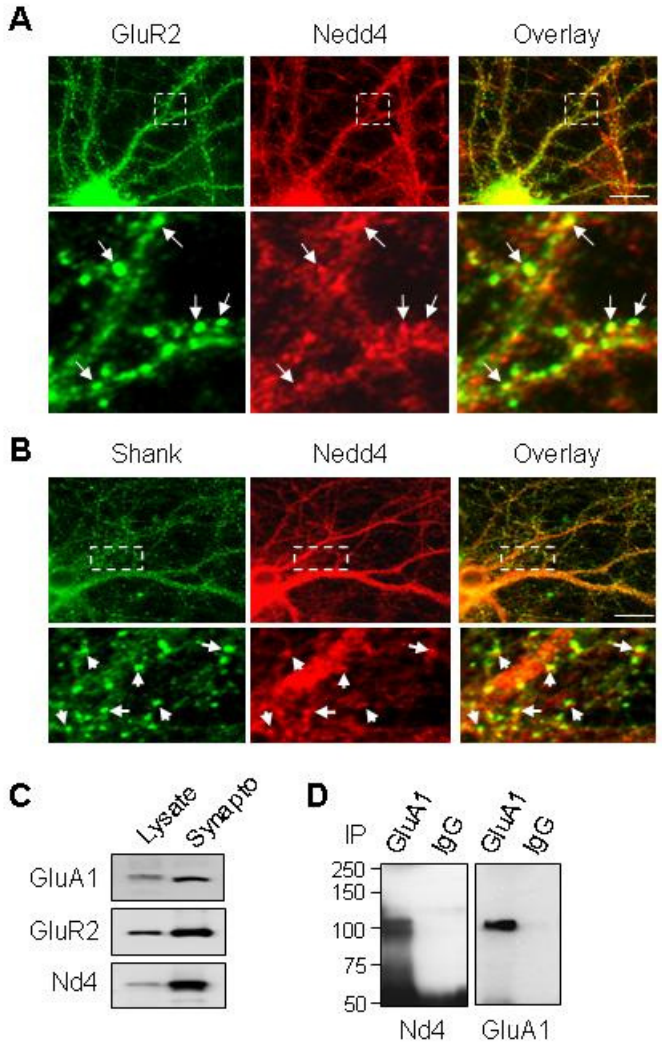
To package lentiviral particles, 6-well plates containing 293T cells at 80-90% confluency were transfected for 4 hours with 3 µg pHDM-Tat 1b (tat accessory protein), 3 µg pRC/CMV-Rev 1b (rev accessory protein), 3 µg pHDM-Hgpm2 (HIV gag-pol expression plasmid), 6 µg pHDM.G (env, VSVG pseudotype) and 12 µg of the target vector (either pHAGE control or pHAGE-Nedd4.1) using Lipofectamine 2000 (Invitrogen) in OPTI-MEM (Gibco). After 4 hours, the transfection solution was replaced with fresh complete DMEM (10% FBS, 1% Pen/Strep). Approximately 48-72H posttransduction, cell debris was removed by centrifugation. One volume of PEG-it Virus Precipitation Solution (System Biosciences) was added to every 4 volumes of supernatant and the virus mixture was allowed to precipitate overnight at 4ºC. The following day, the supernatant was removed after 1500 x *g* centrifugation at 4ºC for 30 minutes. Residual supernatant was removed after an additional spin for 5 minutes. The resultant pellet was resuspended in 200 µl OPTI-MEM and stored at -80ºC. The pHAGE and pHAGE-Nedd4.1 viruses were used at approximately 1:50-1:100 to infect HEK 293A cells at 70% confluency and allowed to incubate without medium change until experiments 48-72 hr post-infection. For neuronal infection, 10-11 DIV cortical neurons were incubated with a similar concentration of Nedd4 virus without medium change until use at DIV 14.

Approximately 2-5 µl of a 109 pfu/ml stock of Ub adenovirus was added to 2.5 ml DMEM/2% FBS, which was then added to a 10 cm dish of 293A cells at 80-90% confluency and incubated for 1.5 hours. Media was replaced with 20 ml DMEM/2%FCS/l-glut. Cells were harvested after 30-48 hrs. Cells were washed once with sterile PBS, collected again at 180 x g for 5 min and then resuspended in 0.2 ml of 10 mM Tris pH 8.1 and freeze/thawed 3-5x to release virus from cells. Lysates were then centrifuged 10 minutes at 4000 x *g* and frozen at -80°C. Ub adenovirus was used at concentrations of 1:500-1:1000 to treat primary neuronal cultures.

Image collection

Immunostained coverslips were mounted onto slides using ProlongGold Antifade reagent (Invitrogen) and kept in the dark for more than 4 hours before imaging. Images were collected with an inverted fluorescence microscope at a 63x oil objective (Zeiss Axiovert 200M). The exposure time for fluorescence signal was first set automatically by the software then adjusted manually so that the signals were within the full dynamic range. Either the glow scale look-up table or the histogram was used to monitor the saturation level. When analyzed using Image J software, images were thresholded to select GluA1 puncta for quantitative measurement. Original images were directly analyzed to assess total protein levels. Image J is a free software available for download from the NIH website (http://rsbweb.nih.gov/ij/download.html).

Supplemental Figure



Supplemental Figure. Nedd4 localizes at the synapse and associates with AMPARs in neurons. Double staining in cortical neurons indicates co-localization of Nedd4 with AMPAR GluA2 (A) or the synaptic protein Shank (B). The boxed area was enlarged (bottom panel) for clarity. Arrows indicate puncta of co-distribution. (C) Western blots of synaptosome fractions prepared from cortical rat brain tissue. Protein assays were performed in lysates prior to westerns to ensure equal loading. AMPAR subunits and Nedd4 were enriched in synaptosomes. (D) Using lysates from rat primary culture, Nedd4 was detected in immunoprecipitates of anti-GluA1 antibodies, but not IgG control (left panel), indicating association of Nedd4 and AMPARs. Reprobing of the membrane confirmed specific pull-down of GluA1 (right panel). Scale bar, 10 μm.